INFECTION AND IMMUNITY, Jan. 2005, p. 298–307 Vol. 73, No. 1
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.1.298–307.2005

Both Innate Immunity and Type 1 Humoral Immunity to *Streptococcus pneumoniae* Are Mediated by MyD88 but Differ in Their Relative Levels of Dependence on Toll-Like Receptor 2

Abdul Q. Khan,[1] Quanyi Chen,[1] Zheng-Qi Wu,[2] James C. Paton,[3] and Clifford M. Snapper[1]*

Department of Pathology, Uniformed Services University of the Health Sciences,[1] and Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health,[2] Bethesda, Maryland, and School of Molecular and Biomedical Sciences, University of Adelaide, South Australia, Australia[3]

Received 12 July 2004/Returned for modification 24 August 2004/Accepted 15 September 2004

Little is known regarding the role of Toll-like receptors (TLRs) in regulating protein- and polysaccharide-specific immunoglobulin (Ig) isotype production in response to an in vivo challenge with an extracellular bacterium. In this report we demonstrate that MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice are markedly defective in their induction of multiple splenic proinflammatory cytokine- and chemokine-specific mRNAs after intraperitoneal (i.p.) challenge with heat-killed *Streptococcus pneumoniae* capsular type 14 (*S. pneumoniae* type 14). This is correlated with analogous responses in splenic cytokine protein release in vitro following addition of *S. pneumoniae* type 14. Consistent with these data, naïve MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice are more sensitive to killing following i.p. challenge with live *S. pneumoniae* type 14, relative to responses in wild-type mice. However, prior immunization of MyD88$^{-/-}$ mice with heat-killed *S. pneumoniae* type 14 protects against an otherwise-lethal challenge with live *S. pneumoniae* type 14. Surprisingly, both MyD88$^{-/-}$ and TLR2$^{-/-}$ mice exhibit striking and equivalent defects in elicitation of type 1 IgG isotypes (IgG3, IgG2b, and IgG2a), but not the type 2 IgG isotype, IgG1, specific for several protein and polysaccharide antigens, in response to i.p. challenge with heat-killed *S. pneumoniae* type 14. Of note, the type 1 IgG isotype titers specific for pneumococcal surface protein A are reduced in MyD88$^{-/-}$ mice but not TLR2$^{-/-}$ mice. These data suggest that distinct TLRs may differentially regulate innate versus adaptive humoral immunity to intact *S. pneumoniae* and are the first to implicate a role for TLR2 in shaping an in vivo type 1 IgG humoral immune response to a gram-positive extracellular bacterium.

Adaptive immunity to extracellular bacteria is largely conferred by antibody. Antibodies specific for both bacterial polysaccharide and protein antigens have been shown to protect the host from infection with otherwise-lethal *Streptococcus pneumoniae* strains (3). Distinct immunoglobulin (Ig) isotypes possess overlapping as well as unique effector functions on the basis of the particular Fc region expressed (45). Thus, the pattern of Ig isotypes elicited during a bacterial infection, in addition to the epitope specificity and affinity of the Ig, might impact on the level of protection afforded by such an antibody. IgG isotypes such as IgG3, IgG2b, and IgG2a, which are associated with Th1 (gamma interferon [IFN-γ]-dominant) immune responses (13, 44, 46), are particularly effective at mediating complement fixation and both complement- and Fc-mediated bacterial opsonophagocytosis. In contrast, IgG1 elicited during Th2 (interleukin-4 [IL-4]-dominant) immune responses may serve a role in neutralizing pathogenic proteins without inducing complement activation and inflammation (45).

Inflammatory cytokines, as well as chemokines, play a key role in innate as well as adaptive host defense against pathogens. Innate immunity, including early cytokine release, is initiated upon recognition of conserved pathogen-associated molecular patterns by various host cells expressing pattern recognition receptors (33). The major pattern recognition receptors in mammalian species are the Toll-like receptor (TLR) family of proteins (2, 42). These proteins share a common cytoplasmic domain with each other, and with the IL-1 and IL-18 receptors, called the Toll-ILRs. Activated Toll-ILRs mediate the eventual translocation of NF-κB and AP-1 into the nucleus via one or a number of distinct adaptor proteins, most critically MyD88, with resultant transcriptional activation of numerous proinflammatory cytokine and chemokine-chemokine receptor genes (19, 48). At present, 11 different TLRs (TLR1 to -11) have been reported, showing distinct specificities for microbial and even host ligands and utilizing different combinations of adaptor proteins (2, 9, 57).

The requirement for TLR2 for signaling in response to peptidoglycan, lipoteichoic acid, and lipoproteins (43) has suggested a dominant role for TLR2 in the induction of innate responses to gram-positive bacteria. Indeed, a key role for TLR2 in mediating innate immunity, including cytokine induction in response to a variety of gram-positive bacteria, including *S. pneumoniae*, has been demonstrated (11, 15, 23, 36, 43, 49). Nevertheless, TLRs other than TLR2 may play a role in responses to gram-positive bacteria. Thus, MyD88$^{-/-}$ mice showed greater lethality and a more profound defect in macrophage cytokine production in response to *Staphylococcus aureus* relative to that in TLR2$^{-/-}$ mice (49). It was recently

* Corresponding author. Mailing address: Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Phone: (301) 295-3490. Fax: (301) 295-1640. E-mail: csnapper@usuhs.mil.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUG 2004		**00-00-2004 to 00-00-2004**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Both Innate Immunity and Type 1 Humoral Immunity to Streptococcus pneumoniae Are Mediated by MyD88 but Differ in Their Relative Levels of Dependence on Toll-Like Receptor 2	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences,Department of Pathology,Bethesda,MD,20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**10**	

shown that although TLR2$^{-/-}$ mice are more susceptible to experimental *S. pneumoniae* meningitis, a substantial part of the inflammatory response was TLR2 independent (23). Additionally, TLR2$^{-/-}$ mice inoculated intranasally with live *S. pneumoniae* displayed only a modestly reduced inflammatory response in the lungs and normal host immunity relative to that in wild-type mice, despite defective cytokine production from freshly isolated TLR2$^{-/-}$ alveolar macrophages (22). Less is known regarding the role of TLRs in shaping the adaptive humoral response to an intact pathogen. Injection of mice with purified antigens in adjuvant demonstrated a role for MyD88 in mediating an antigen-specific type 1, but not type 2, in vivo IgG isotype response (42). More recently, a normal pathogen-specific IgG isotype response to *Borrelia burgdorferi* was observed in TLR2$^{-/-}$ mice, although this was associated with a higher burden of pathogen (54). However, MyD88$^{-/-}$ mice infected with *B. burgdorferi* demonstrated elevated *B. burgdorferi*-specific IgM and IgG1 responses, but diminished type 1 antibodies (IgG2a, IgG2b, and IgG3) (27). Similarly, MyD88$^{-/-}$ mice exhibited reduced leishmania-specific IgG2a, and elevated IgG1, in response to infection with *Leishmania major* (8). Finally, a recent report demonstrated a role for endogenous TLR2 in stimulating an Ig response to a *Haemophilus influenzae* type b-outer membrane protein complex (OMPC) glycoconjugate vaccine that was specific for both the *H. influenzae* capsular polysaccharide and the OMPC protein (26).

We earlier demonstrated that challenge of wild-type mice with intact *S. pneumoniae*, a gram-positive extracellular bacterium, rapidly induces (by 2 to 6 h) a number of proinflammatory cytokines in the spleen (20). We further demonstrated that the endogenous release of tumor necrosis factor alpha (TNF-α), IL-6, and IFN-γ independently stimulated both IgG antiprotein and antipolysaccharide responses of various isotypes following *S. pneumoniae* immunization. Additionally, both IgG antiprotein and IgG antipolysaccharide responses to *S. pneumoniae* were dependent on T-cell receptor α/β^+ CD4$^+$ T cells (21, 55), B7-dependent costimulation (55, 56), and dendritic cells (6, 7). Direct TLR signaling, including the subsequent release of cytokines, induces dendritic cells to migrate to secondary lymphoid organs, upregulate T-cell costimulatory molecules, including B7, and release IL-12, thus leading to the priming of CD4$^+$ T cells for type 1 immunity. In this regard, we postulated that TLRs would play an important role in the in vivo antiprotein and antipolysaccharide Ig isotype response to intact *S. pneumoniae*. In this report, we demonstrate a MyD88-dependent pathway for innate protection against challenge with *S. pneumoniae* which is largely independent of TLR2 but, surprisingly, we observed that both the protein- and polysaccharide-specific type 1 IgG isotype response is largely TLR2 dependent. These data are of particular interest in light of earlier contrasting reports suggesting that TLR2 stimulation favors type 2 responses (10, 39, 40).

MATERIALS AND METHODS

Mice. TLR2$^{-/-}$ mice (129B6 background) (47) and MyD88$^{-/-}$ mice (C57BL/6 background) (1) were obtained originally from S. Akira (Osaka University, Osaka, Japan) and were bred in our facility. 129B6 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). C57BL/6 mice were obtained from the National Cancer Institute (Frederick, Md.). All mice were typically used between 6 and 10 weeks of age. These studies were conducted in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, revised 1996 (34a), and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Use and Care Committee.

Genotyping. (i) TLR2$^{-/-}$ mice. The following primers were used for genotyping TLR2$^{-/-}$ mice: primer A, 5′ GTT TAG TGC CTG TAT CCA GTC AGT GCG 3′; B, 5′ AAT GGG TCA AGT CAA CAC TTC TCT GGC 3′; and C, 5′ ATC GCC TTC TAT CGC CTT CTT GAC GAG 3′. For PCR, we used 200 ng of DNA and applied the following conditions: 94°C for 1 min; 35 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Products were separated on a 1% agarose gel. For detection of the mutated allele, we used primers B and C. For the wild-type allele, we used primers A and B. The amplified products were both about 1,200 bp.

(ii) MyD88$^{-/-}$mice. For genotyping of MyD88$^{-/-}$ mice, three primers were mixed together: left, 5′ TGG CAT GCC TCC ATC ATA GTT AAC C 3′; right, 5′ GTC AGA AAC AAC CAC CAC CAT GC 3′; and *Neo*, 5′ ATC GCC TTC TAT CGC CTT CTT GAC G 3′. For PCR, we used 200 ng of DNA and applied the following conditions: 94°C for 3 min; 35 cycles of 94°C for 40 s, 65°C for 40 s, and 72°C for 50 s; and 72°C for 10 min. Products were separated on a 2% Nusieve agarose gel. The wild-type gene gave a product 563 bp long. The size of the product in knockout mice was about 640 bp.

Reagents. Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* BJ3505 as a His_6-tagged fusion protein and purified by nickel-nitrilotriacetic acid affinity chromatography (55). PsaA and PspC (CbpA) were expressed as N-terminal His_6-fusion proteins in recombinant *Escherichia coli* cells and purified by nickel-nitrilotriacetic acid affinity chromatography, as previously described (35, 38). The purified proteins were >95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after staining with Coomassie brilliant blue R250. Phosphorylcholine-keyhole limpet hemacyanin (PC-KLH) was synthesized as described previously (55). The resulting conjugate had a substitution degree of 19 PC/KLH molecule. Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from the American Type Culture Collection (Manassas, Va.).

Preparation and immunization of *S. pneumoniae* type 14. A frozen stock of *S. pneumoniae* capsular type 14 (*S. pneumoniae* type 14) was thawed and subcultured on BBL premade blood agar plates (VWR International, Bridgeport, N.J.). Isolated colonies on blood agar were grown in Todd-Hewitt broth (Becton Dickinson, Sparks, Md.) to mid-log phase, collected, and heat killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with phosphate-buffered saline (PBS) to give an absorbance reading at 650 nm of 0.6, which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at −80°C until used as antigen for mouse immunizations. Mice were immunized intraperitoneally (i.p.) with 2×10^8 CFU of heat-killed bacteria in 250 μl of PBS. The *S. pneumoniae* type 14 stock was tested for endotoxin using the *Limulus* amebocyte lysate assay (QCL-1000) from BioWhittaker (Walkersville, Md.). This assay demonstrated that mice injected with 2×10^8 CFU equivalents of *S. pneumoniae* type 14 received <20 pg of endotoxin (if at all). Serum samples for measurement of anti-PPS14, anti-PC, anti-PspA, anti-PsaA, and anti-PspC antibody titers were prepared from blood obtained through the tail vein.

Real-time reverse transcription-PCR (RT-PCR) for measurement of splenic cytokine-specific mRNA ex vivo. Total RNA was extracted from isolated spleen cell populations using RNAzol B (TEL-TEST, Inc., Friendswood, Tex.). Total RNA was then reverse transcribed using the Superscript II preamplification system for first-strand cDNA synthesis (Invitrogen) according to the manufacturer's instructions. A 180-ng aliquot of RNA was subsequently used as template for each real-time PCR. All PCRs for cytokine-specific mRNA were performed on an ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, Calif.) using proprietary cytokine-specific primers and probes from ABI Applied Biosystems (Rockville, Md.). Relative cytokine mRNA levels were determined by normalization of signal with that for rRNA. In initial studies, twofold dilutions of cDNA generated a linear signal curve over at least a 30-fold range of cDNA concentrations.

Measurement of cytokine concentrations in spleen cell culture supernatant (SN). The concentrations of specific cytokines released into the media of spleen cell cultures to which *S. pneumoniae* type 14 was added were measured using optimized standard sandwich enzyme-linked immunosorbent assay (ELISA). Recombinant cytokines used as standards, as well as the capture monoclonal antibodies, biotinylated monoclonal antibodies used for detection, and streptavidin-alkaline phosphatase (AP) were purchased from BD Pharmingen (San Diego, Calif.). Streptavidin-AP was used in combination with *p*-nitrophenyl

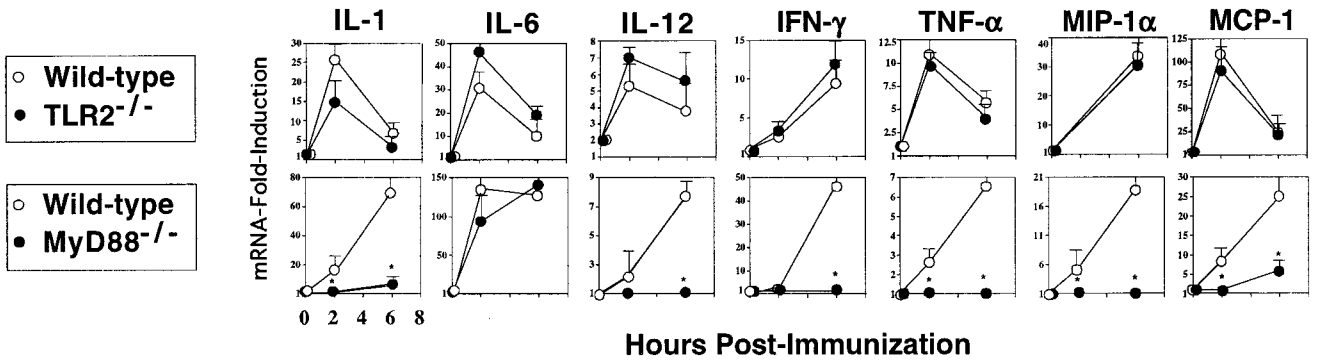

FIG. 1. MyD88$^{-/-}$ mice, but not TLR2$^{-/-}$ mice, exhibit reduced splenic cytokine and chemokine mRNA induction in response to i.p. immunization with intact heat-killed *S. pneumoniae* type 14 relative to wild-type controls. MyD88$^{-/-}$ and wild-type C57BL/6 mice (five mice per group) and TLR2$^{-/-}$ and wild-type 129B6 mice (five per group) were immunized i.p. with heat-killed *S. pneumoniae* type 14 (2×10^8 CFU/mouse). Spleens were removed from naïve (unimmunized) mice (five per group) and from mice at 2 and 6 h following immunization. RNA was purified, and real-time RT-PCR was performed to determine the relative expression levels of selected cytokine and chemokine mRNAs by using rRNA to standardize individual samples. The arithmetic mean of specific mRNA levels in spleen cells obtained from individual unimmunized mice was arbitrarily assigned a value of 1. Values represent the arithmetic mean ± SEM. *, $P \leq 0.05$. Data are representative of two independent experiments.

phosphate, disodium (Sigma, St. Louis, Mo.) as substrate to detect the specific binding. Standards were included in every plate, and the samples were tested in duplicate.

Measurement of serum antigen-specific Ig isotype titers. Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, Va.) were coated (50 μl/well) with either PC-KLH (5 μg/ml), PPS14 (5 μg/ml), PspA (5 μg/ml), PsaA (1 μg/ml), or PspC (1 μg/ml) in PBS for 1 h at 37°C or overnight at 4°C. Plates were washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1% bovine serum albumin for 30 min at 37°C or overnight at 4°C. Threefold dilutions of serum samples, starting at a 1/50 serum dilution in PBS plus 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C, and plates were washed three times with PBS plus 0.1% Tween 20. AP-conjugated polyclonal goat anti-mouse IgM or IgG antibodies (200 ng/ml final concentration in PBS plus 0.05% Tween 20) were then added, and plates were incubated at 37°C for 1 h. Plates were washed five times with PBS plus 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer (1 M Tris plus 0.3 mM $MgCl_2$; pH 9.8) was then added for 30 min at room temperature for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems). No cross-reactivity was observed between the serum titers of anti-PspA, anti-PspC, and anti-PsaA using the respective recombinant proteins in the dilution buffer, where only the relevant protein mediated blocking (data not shown). Further, addition of C-polysaccharide (10 μg/ml) plus Cps22F (20 μg/ml) into the serum dilution buffer did not inhibit signal generation in the IgG anti-PPS14 ELISA and inhibited <10% of the signal generated in the IgM anti-PPS14 ELISA. Sera from R36A (unencapsulated variant of type 2 *S. pneumoniae*)-immunized mice also did not react in the anti-PPS14 ELISA. Thus, these blocking reagents were not routinely used in this assay.

Lethality studies. Live *S. pneumoniae* type 14 cells were grown to mid-log phase. Bacterial numbers were determined by colony counts on blood agar (Becton Dickinson, Cockeysville, Md.). Mice were injected i.p. with either 2×10^8, 1×10^8, 4×10^7, and/or 2×10^7 live *S. pneumoniae* type 14 in 250 μl of PBS. Mice were observed daily for up to 7 days after infection.

Statistics. Data were expressed as arithmetic means ± standard errors of the means (SEM) of the individual titers. Levels of significance of the differences between groups were determined by the Student *t* test. *P* values of ≤0.05 were considered statistically significant.

RESULTS

MyD88$^{-/-}$ mice, but not TLR2$^{-/-}$ mice, are defective in splenic cytokine and chemokine induction in response to i.p. immunization with intact heat-killed *S. pneumoniae* type 14. We utilized mice genetically deficient in TLR2 (TLR2$^{-/-}$) (47) to first determine the early splenic cytokine response to *S. pneumoniae*. We utilized heat-killed *S. pneumoniae* type 14 diluted in PBS, which we previously demonstrated induces peak inflammatory splenic cytokine mRNA levels between 2 and 6 h following i.p. immunization (20). Injection of PBS alone had no effect (data not shown). TLR2$^{-/-}$ and wild-type mice were immunized i.p. with heat-killed *S. pneumoniae* type 14, and spleens were removed from either naïve mice or from mice at either 2 or 6 h following immunization for determination of relative levels of selected inflammatory cytokine and chemokine mRNAs by real-time RT-PCR. No significant differences were observed between TLR2$^{-/-}$ and wild-type mice in the relative induction of mRNA specific for IL-1, IL-6, IL-12, IFN-γ, TNF-α, macrophage inflammatory protein 1α (MIP-1α), or monocyte chemotactic protein 1 (MCP-1) at either 2 or 6 h following immunization (Fig. 1, upper panel).

MyD88 is an adaptor protein that plays a critical role in signaling via all TLRs, as well as through the IL-1R and IL-18R, including signals required for cytokine induction (1, 19, 48). Of note, TLR3 and TLR4 can signal immune cells in a MyD88-independent manner, utilizing another TLR adaptor designated Ticam-1 or Trif, of particular importance for induction of type 1 IFN (17, 18). Thus, we utilized MyD88$^{-/-}$ mice (1) to determine whether a TLR other than, or in addition to, TLR2 was required for in vivo cytokine induction in response to *S. pneumoniae* type 14. As with TLR2$^{-/-}$ mice, MyD88$^{-/-}$ and wild-type mice were immunized i.p. with heat-killed *S. pneumoniae* type 14 and spleens were removed from either naïve mice or mice at either 2 or 6 h following immunization for real-time RT-PCR analysis. MyD88$^{-/-}$ mice were markedly defective in their induction of splenic mRNA specific for IL-1, IL-12, IFN-γ, TNF-α, MIP-1α, and MCP-1 at both 2 and 6 h postimmunization. In notable contrast, MyD88$^{-/-}$ and wild-type mice consistently exhibited equivalent induction of IL-6 mRNA in response to *S. pneumoniae* type 14 (Fig. 1, lower panel).

To determine whether splenic mRNA induction in response to *S. pneumoniae* type 14 in vivo correlated with splenic cytokine protein release in wild-type versus TLR2$^{-/-}$ or MyD88$^{-/-}$ mice, we stimulated whole spleen cells in vitro with

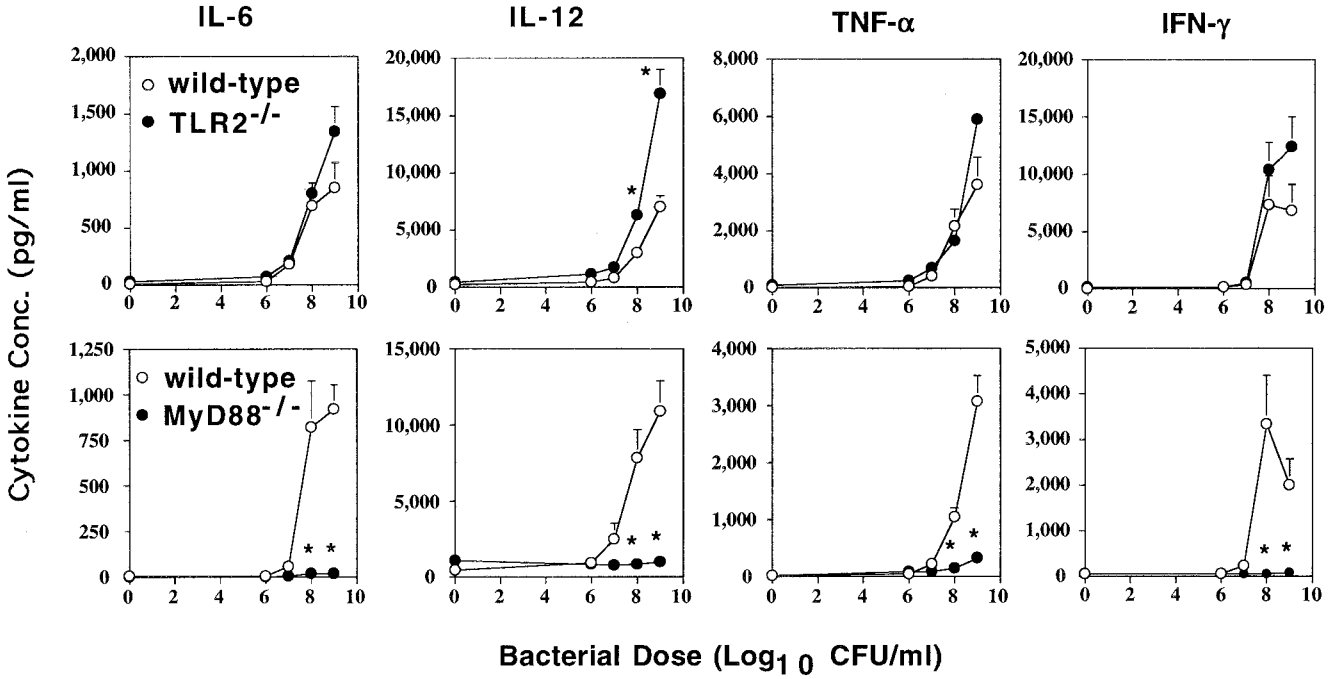

FIG. 2. Spleen cells from MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice exhibit reduced cytokine secretion in response to in vitro stimulation with heat-killed *S. pneumoniae* type 14 relative to wild-type controls. Spleen cells (10^7 cells/ml) from wild-type (C57BL/6 and 129B6), MyD88$^{-/-}$, and TLR2$^{-/-}$ (three per group) mice were cultured with different doses of heat-killed *S. pneumoniae* type 14 (10^9, 10^8, 10^7, and 10^6 CFU/ml) for 24 h. Concentrations of IL-6, IL-12, TNF-α, and IFN-γ in the culture supernatants were then determined by ELISA. Values represent the arithmetic mean ± SEM. *, $P \leq 0.05$. The results are representative of two independent experiments.

various concentrations of heat-killed *S. pneumoniae* type 14 and measured the SN concentrations of IL-6, IL-12, TNF-α, and IFN-γ 24 h later by ELISA. Wild-type and TLR2$^{-/-}$ spleen cells secreted equivalent amounts of all four cytokines in a dose-dependent manner and at each dose of *S. pneumoniae* type 14 (Fig. 2). In contrast, MyD88$^{-/-}$ spleen cells failed to secrete detectable IL-12, TNF-α, IFN-γ, and even IL-6 at any dose of *S. pneumoniae* type 14. Thus, with the exception of IL-6 in MyD88$^{-/-}$ mice, the changes observed in splenic cytokine mRNA and cytokine protein levels after challenge with *S. pneumoniae* type 14 were consistent. The potential mechanism(s) and significance underlying the differences observed between splenic IL-6 mRNA and protein are discussed below.

MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice are more sensitive to killing, relative to wild-type mice, after i.p. challenge with live *S. pneumoniae* type 14. The early release of proinflammatory cytokines during the innate response to bacteria is important in mediating bacterial clearance prior to the onset of adaptive, humoral immunity (20). Although TLR2$^{-/-}$ mice appeared to make a largely normal splenic cytokine and chemokine response to challenge with *S. pneumoniae* type 14, the number of cytokines and chemokines tested were limited and only the spleen was evaluated, thus raising the possibility of mediator and/or site-selective, but critical, defects in their innate response to *S. pneumoniae* type 14. An analogous, though converse, argument could also be made concerning MyD88$^{-/-}$ mice.

Thus, we performed lethality studies in naïve wild-type versus TLR2$^{-/-}$ and MyD88$^{-/-}$ mice using live *S. pneumoniae* type 14 to assess the overall effectiveness of their innate responses. Initially, we infected naïve, wild-type mice i.p. with various doses of live *S. pneumoniae* type 14 (five animals per group). Doses of 2×10^8 and 1×10^8 CFU were uniformly fatal, only one of five mice was killed with 4×10^7 CFU, and no mice died when injected with 2×10^7 CFU of *S. pneumoniae* type 14 (Fig. 3A). We then infected naïve wild-type and MyD88$^{-/-}$ mice (five per group) i.p. with either 4×10^7 or 2×10^7 CFU of live *S. pneumoniae* type 14. No wild-type mice died over a 6-day period when infected with either 4×10^7 CFU or 2×10^7 CFU of *S. pneumoniae* type 14. In contrast, all MyD88$^{-/-}$ mice died during this period when infected with 4×10^7 CFU (all by day 4) or 2×10^7 CFU (all by day 6) (Fig. 3B). To determine whether adaptive immunity could protect MyD88$^{-/-}$ mice from otherwise-lethal *S. pneumoniae* type 14 infection, we first immunized wild-type and MyD88$^{-/-}$ mice (eight per group) i.p. with 2×10^8 heat-killed *S. pneumoniae* type 14 and similarly boosted them on day 14 (see also Fig. 5 and 6, below). On day 28, immunized mice were infected i.p. with 4×10^7 CFU of live *S. pneumoniae* type 14 and compared with their similarly infected naïve counterparts (eight animals per group). Neither naïve nor immunized wild-type mice were killed by *S. pneumoniae* type 14 over a 5-day period (Fig. 3C). Although all of the naïve MyD88$^{-/-}$ mice were killed by *S. pneumoniae* type 14 by day 4, none of the immunized MyD88$^{-/-}$ mice succumbed to infection over a 5-day period. In contrast to naïve MyD88$^{-/-}$ mice, naïve TLR2$^{-/-}$ mice were found to be at most only slightly more vulnerable to killing with live *S. pneumoniae* type 14 than wild-type mice (Fig. 3D). Like MyD88$^{-/-}$ mice, prior immunization of

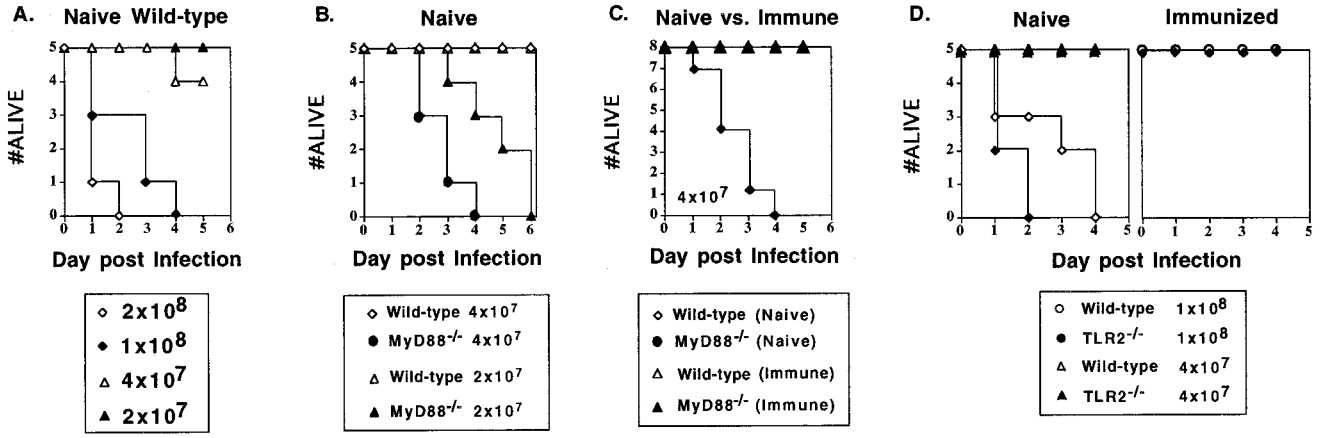

FIG. 3. MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice are more sensitive to killing, relative to wild-type mice, after i.p. challenge with live *S. pneumoniae* type 14. Naïve wild-type (C57BL/6 and 129B6), MyD88$^{-/-}$, and TLR2$^{-/-}$ mice were inoculated i.p. with different doses of live *S. pneumoniae* type 14 and were monitored for survival. A separate group of mice were first challenged with heat-killed *S. pneumoniae* type 14 (2×10^8 CFU/mouse) i.p. and then rechallenged with live *S. pneumoniae* type 14 i.p. 3 weeks later. Results are from five (groups A, B, and D) and eight (group C) mice per group.

TLR2$^{-/-}$ mice with heat-killed *S. pneumoniae* type 14 protected them from an otherwise-lethal dose of live *S. pneumoniae* type 14 (Fig. 3D). These data demonstrate a marked defect in innate host protection to live *S. pneumoniae* type 14 in MyD88$^{-/-}$ mice that is likely secondary to a severely blunted early proinflammatory cytokine response. The similar induction of cytokines observed in TLR2$^{-/-}$ and wild-type mice is consistent with their largely similar resistance to live *S. pneumoniae* type 14 challenge.

Both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice exhibit a defective in vivo type 1 IgG isotype antipolysaccharide, and antiprotein, response to heat-killed *S. pneumoniae* type 14. Immunization of wild-type mice with intact heat-killed *S. pneumoniae* type 14 induces a primary IgM and IgG isotype response specific for protein (pneumococcal surface protein A [PspA]) and polysaccharide antigens (PC determinant of cell wall teichoic acid and serotype-specific capsular polysaccharide [PPS14]) (21). Whereas the IgM anti-PC and anti-PPS14 response to *S. pneumoniae* type 14 is T-cell independent, induction of IgG anti-PC, anti-PPS14, and anti-PspA is dependent on CD4$^+$ T cells. Boosting of *S. pneumoniae* type 14-primed mice leads to substantial increases in both IgG anti-PspA as well as IgG anti-PC responses, but no further increases in IgG anti-PPS14 (21). Further, endogenous releases of proinflammatory cytokines (e.g., TNF-α, IL-6, and IFN-γ) are independently stimulatory for both anti-PspA and anti-PC responses (20). In light of an earlier finding that immunization of MyD88$^{-/-}$ mice with purified antigens in adjuvant leads to defective type 1, but not type 2, T-cell priming and subsequent IgG isotype production (42), we wished to determine the IgM and IgG isotype antipolysaccharide and antiprotein responses to intact *S. pneumoniae* type 14 in both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice.

TLR2$^{-/-}$ and wild-type mice were immunized i.p. with heat-killed *S. pneumoniae* type 14 and similarly boosted 14 days later. Surprisingly, TLR2$^{-/-}$ mice exhibited a significant reduction in type 1 IgG isotypes (i.e., IgG3, IgG2b, and IgG2a) specific for PC and PPS14 in both the primary and secondary response (Fig. 4). More modest reductions in serum IgM anti-PC and anti-PPS14 titers in TLR2$^{-/-}$ mice were observed, although not at all time points. In contrast, both TLR2$^{-/-}$ and wild-type mice elicited a similar type 2, IgG1 anti-PC and anti-PPS14 response. Of note, the anti-PspA response in TLR2$^{-/-}$ mice was comparable to that observed in wild-type mice for all IgG isotypes (Fig. 4).

As further illustrated in Fig. 5, MyD88$^{-/-}$ mice, relative to wild-type mice, immunized and boosted i.p. with heat-killed *S. pneumoniae* type 14 also had significant decreases in type 1 IgG isotype responses (IgG3, IgG2a, and IgG2b) specific for PC and PPS14 to an extent comparable to that observed in TLR2$^{-/-}$ mice. The type 2 IgG isotype (IgG1) was, in contrast, significantly higher in MyD88$^{-/-}$ versus wild-type mice for both PC and PPS14, although not at all time points. Only a variably modest reduction in IgM anti-PC was observed in MyD88$^{-/-}$ mice. These overall differences were observed for both the primary and secondary responses. In contrast to TLR2$^{-/-}$ mice, MyD88$^{-/-}$ mice showed a defective type 1, although normal type 2, IgG isotype response specific for PspA (Fig. 5). Although the preimmune serum titers of IgM anti-PPS14 appeared to be different between control C57BL/6 and B6I29 mice, analysis of these serum samples on the same set of ELISA plates indicated equivalent preimmune titers (data not shown). Given the relative nature of serum titer calculations, sera from each experiment were analyzed using the same set of ELISA plates, allowing for the accurate, direct comparisons between different experimental groups.

In a final set of experiments, we wished to determine whether the differences observed between TLR2$^{-/-}$ and MyD88$^{-/-}$ mice in the IgG anti-PspA response represented a more generalized difference in the overall antiprotein response between these two strains of mice. Thus, we further analyzed the sera from the experiments illustrated in Fig. 4 and 5 for serum IgG isotype titers specific for two additional proteins, PspC (CbpA) (pneumococcal surface protein C or choline-binding protein A) and PsaA (pneumococcal surface antigen A). The former is bound to PC, which is linked to the cell wall C-polysaccharide (teichoic acid), whereas the latter is a membrane-bound lipoprotein. Of note, whereas strong primary IgG anti-PspA responses were typically observed in both the

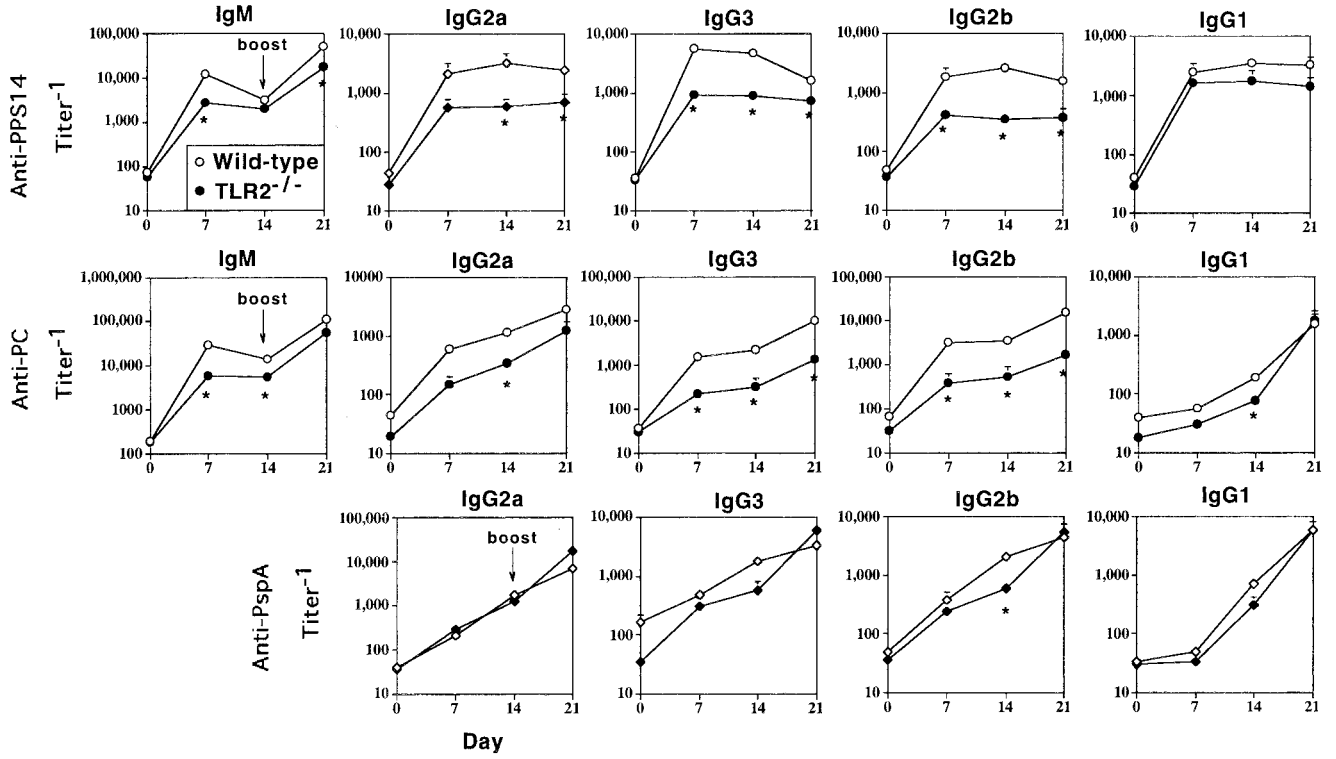

FIG. 4. TLR2$^{-/-}$ mice have a reduced type 1 IgG anti- PPS14 and anti-PC, but not anti-PspA, response to heat-killed *S. pneumoniae* type 14 relative to wild-type controls. TLR2$^{-/-}$ and control 129B6 mice (seven per group) were immunized i.p. with 2×10^8 CFU of heat-killed *S. pneumoniae* type 14 and then similarly boosted on day 14. Serum titers of PPS14-, PC- and PspA-specific Ig isotypes (days 0, 7, 14, and 21) were determined by ELISA. Values represent the arithmetic mean ± SEM. *, $P \leq 0.05$. One of two representative experiments is shown.

C57BL/6 and 129B6 wild-type mice challenged with heat-killed *S. pneumoniae* type 14, we observed very weak primary IgG anti-PspC and IgG anti-PsaA responses in these two wild-type strains (data not shown). However, upon boosting of primed mice with *S. pneumoniae* type 14, a strong induction of IgG anti-PspC and IgG anti-PsaA was obtained. In striking contrast to what we observed for IgG anti-PspA, secondary type 1, but not type 2, IgG isotype titers specific for PspC and PsaA were significantly reduced in both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice (Fig. 6). Collectively, these data indicate that TLR2 plays a dominant role in the induction of type 1 humoral immunity specific for both proteins and polysaccharide antigens in response to intact, heat-killed *S. pneumoniae* type 14, whereas the innate response, though MyD88 dependent, can be effectively mediated through a TLR(s) other than TLR2.

DISCUSSION

TLR2 has been implicated as an important innate recognition receptor for gram-positive extracellular bacteria (36, 43, 49, 52), and it independently plays a significant though variable role in innate protection against *S. pneumoniae*, possibly dependent upon the initial site of infection (11, 20, 31). However, little is known concerning the role of TLR2, or that of the general TLR adaptor protein MyD88, in shaping in vivo antiprotein and antipolysaccharide Ig isotype responses to *S. pneumoniae* or other intact gram-positive extracellular bacteria. In this report we demonstrate a critical role for MyD88 in mediating innate cytokine release and early protection following i.p. injection of intact *S. pneumoniae*, although no apparent, independent role was observed for TLR2. However, both MyD88$^{-/-}$ and TLR2$^{-/-}$ mice showed substantial deficiencies in elicitation of type 1 IgG isotypes (i.e., IgG3, IgG2b, and IgG2a) specific for a number of protein and polysaccharide antigens in response to immunization with intact *S. pneumoniae*. A notable exception was the normal type 1 IgG anti-PspA response observed in TLR2$^{-/-}$, but not MyD88$^{-/-}$, mice. In contrast, we demonstrated either normal or elevated IgG1 (type 2) antiprotein and antipolysaccharide responses in both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice. IgM antipolysaccharide responses in TLR2$^{-/-}$ and MyD88$^{-/-}$ mice were only modestly reduced. These studies leave open the additional possibility of a role for either TLR1 and/or TLR6 in regulating the TLR2-dependent induction of the *S. pneumoniae* type 14-induced type 1 IgG response, in light of the critical role for these former TLRs as coreceptors for certain TLR2 ligands (36, 50, 51).

These data are the first to implicate a role for TLR2 in shaping a type 1 IgG humoral immune response to an extracellular bacterium, although the mechanism underlying this defect is currently unknown. In this regard, i.p. immunization with *S. pneumoniae* induces early splenic IL-12, IFN-γ, MIP-1α, and MCP-1 mRNA expression in TLR2$^{-/-}$ mice that is quantitatively similar to that in wild-type mice at both 2 and 6 h postimmunization. Secretion of both IL-12 and IFN-γ protein from TLR2$^{-/-}$ and wild-type spleen cells stimulated with *S.*

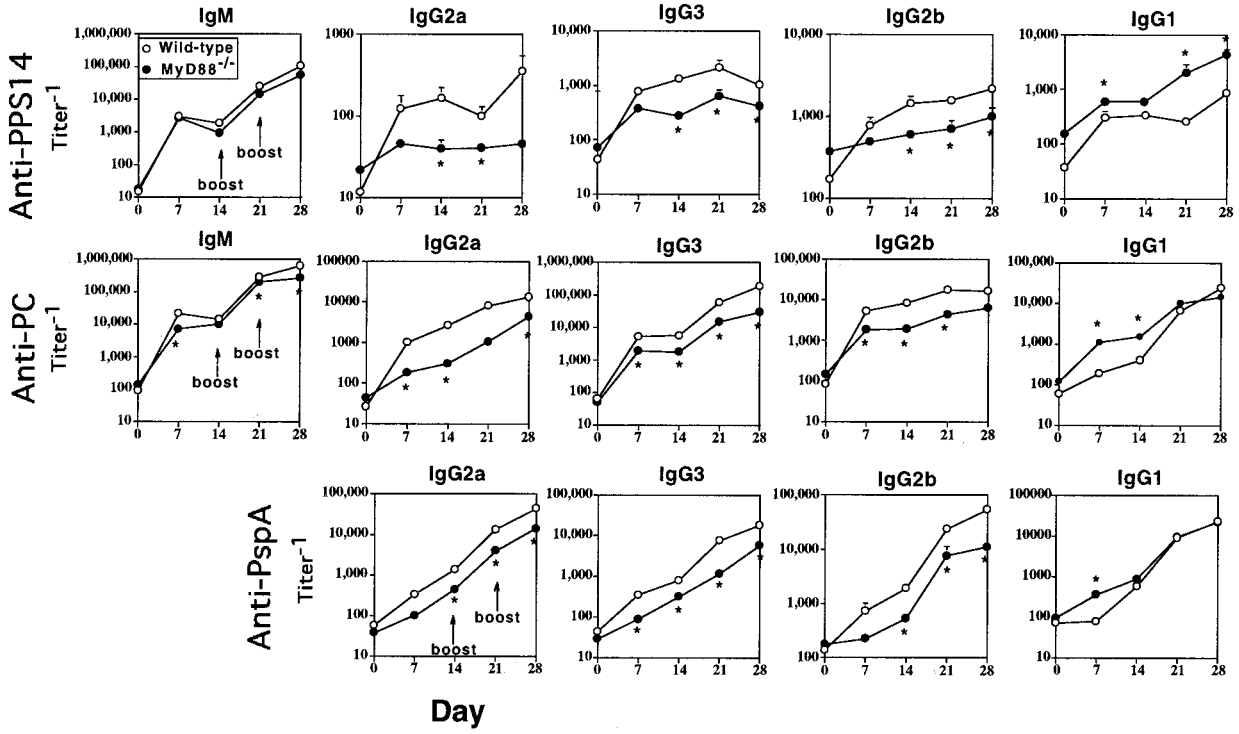

FIG. 5. MyD88$^{-/-}$ mice have a reduced type 1 IgG anti-PPS14, anti-PC, and anti-PspA response to heat-killed *S. pneumoniae* type 14 relative to wild-type controls. MyD88$^{-/-}$ and wild-type (C57BL/6) mice (eight per group) were each immunized i.p. with heat-killed *S. pneumoniae* type 14 (2×10^8 CFU/mouse) and then boosted on days 14 and 21 with the same dose. Serum titers of PPS14-, PC- and PspA-specific Ig isotypes (days 0, 7, 14, 21, and 28) were determined by ELISA. Values represent the arithmetic mean ± SEM. *, $P \leq 0.05$. One of three representative experiments is shown.

pneumoniae in vitro was also comparable. IL-12, IFN-γ, and MIP-1α have been independently implicated in promoting a type 1 adaptive immune response, whereas MCP-1 promotes type 2 immunity (29, 34). Of note, our analyses of cytokine and chemokine induction were limited to a select number of key proinflammatory cytokines and chemokines, leaving open the likely prospect that a selective defect in TLR2$^{-/-}$ mice that is critical for promotion of type 1 immunity will be revealed by a more comprehensive analysis of mediator expression. We believe it unlikely that kinetic differences in mediator release underlie the defective type 1 humoral response, since we previously demonstrated that splenic cytokine mRNA expression returns to baseline by 12 h in wild-type mice and remains at baseline levels up to 3 days following immunization (20).

Our analyses of the TLR dependence of the innate and humoral responses to i.p. challenge with *S. pneumoniae* involved immunization of mice with heat-killed *S. pneumoniae* type 14. Heat-killing of *S. pneumoniae* destroys pneumolysin activity, which is otherwise present in live *S. pneumoniae* (7). Pneumolysin was recently shown to be a TLR4 ligand capable of stimulating IL-6 and TNF-α from macrophages (31). In this regard, TLR4$^{-/-}$ mice were shown to be more susceptible to lethality following intranasal colonization with pneumolysin-positive *S. pneumoniae* (31). Although our lethality studies necessarily utilized live *S. pneumoniae* expressing pneumolysin, we believe that our logical correlations with innate cytokine production using heat-killed *S. pneumoniae* are still valid. Thus, TLR2$^{-/-}$ mice, unlike MyD88$^{-/-}$mice, exhibited largely normal innate responses to i.p. injection of heat-killed *S. pneumoniae*, even in the absence of pneumolysin, suggesting other redundant, non-TLR2-dependent pathways. Further, TLR4-mediated cytokine induction, as would be observed using pneumolysin, is also MyD88 dependent, suggesting that cytokine profiles between heat-killed and live *S. pneumoniae* are likely to be similar in MyD88$^{-/-}$ mice. A possible exception is the potential for TLR4-dependent, MyD88-independent effects, such as type 1 interferon induction (17, 18). Our demonstration of defective type 1 humoral immunity in TLR2$^{-/-}$ mice, using heat-killed *S. pneumoniae*, further indicates that in the absence of pneumolysin, and possibly other heat-sensitive TLR ligands, TLR2-mediated signaling alone can be critical for induction of TLR-dependent type 1 humoral immunity. However, these data leave open the possibility that an additional TLR(s) and its ligand(s) (e.g., TLR4 and pneumolysin) may play a redundant role in Ig isotype induction in response to live *S. pneumoniae*.

The striking differences observed in the induction of the early cytokine response in TLR2$^{-/-}$ versus MyD88$^{-/-}$ mice by using heat-killed *S. pneumoniae* type 14 implicate a TLR ligand other than TLR2 in the innate response to intact *S. pneumoniae*. Although heat-killing of *S. pneumoniae* resulted in loss of TLR4-dependent pneumolysin activity, continued expression of an additional, heat-stable TLR4 ligand, currently unappreciated, is possible. Another possibility is that unmethylated CpG-containing DNA in the *S. pneumoniae* genome mediates TLR9 signaling, known to be entirely MyD88 depen-

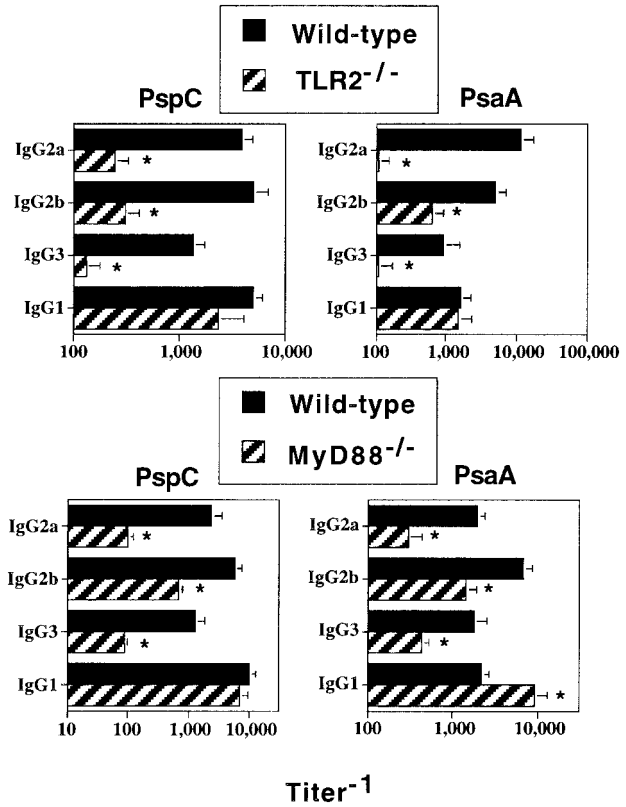

FIG. 6. Both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice have a reduced in vivo type 1 IgG anti-PspC and anti-PsaA response to heat-killed *S. pneumoniae* type 14 relative to wild-type controls. Serum samples were from mice described in the legends for Fig. 4 and 5. Day 21 sera were analyzed for determination of antigen-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM. *, $P < 0.05$. One of two representative experiments is shown.

dent (16). Although most studies on TLR9 signaling have used synthetic unmethylated CpG oligodeoxynucleotides, several studies have suggested an important role for TLR9 signaling in response to intact pathogens, such as viruses (e.g., herpes simplex type 1 and type 2 and human immunodeficiency virus type 1) (12, 25, 28). Additionally, TLR9$^{-/-}$ mice were shown to have a defective antigen-specific Th1 response in response to *Candida albicans* (4). Of interest, malaria schizonts were shown to activate plasmacytoid dendritic cells via TLR9 to secrete IFN-α. However, a schizont-soluble fraction that had this activity was heat labile and precipitated by ammonium sulfate, indicating that the active component was not DNA (37). Recently, it was demonstrated that single-stranded RNA, especially that containing poly(G) or poly(U) and poly(G), can signal immune cells via murine TLR7 or human TLR8 (9, 15). Of interest, single-stranded RNA from *Streptococcus pyogenes* and *Enterococcus faecalis* was able to induce IL-12 when transfected into human monocyte-derived dendritic cell precursors costimulated with CD40 ligand; eukaryotic RNA did not have this effect (24).

Our observation that endogenous TLR2 signaling in response to heat-killed *S. pneumoniae* is necessary for induction of type 1 humoral immunity stands in contrast to previous studies that suggested a key role for TLR2 in elicitation of type 2 immunity (10, 39, 40). In one study, coinjection of the TLR2 ligand Pam_3Cys with ovalbumin (OVA) was shown to augment a type 2 response and exacerbate disease in a model of experimental asthma, whereas TLR9 signaling in response to a CpG-containing oligodeoxynucleotide promoted a type 1 response and ameliorated disease (40). Similarly, adoptive transfer of OVA-specific transgenic CD4$^+$ T cells and OVA peptide in combination with Pam_3CyS versus *E. coli* lipopolysaccharide (LPS; a TLR4 ligand) into wild-type mice followed by OVA restimulation of spleen cells in vitro resulted in induction of lower levels of IFN-γ and higher levels of IL-4 and IL-5 when using Pam_3CyS relative to that with *E. coli* LPS, the latter instead inducing a type 1 cytokine response (10). Similar results were observed when using *Porphyromonas gingivalis* LPS (a TLR2 ligand) instead of Pam_3Cys versus *E. coli* LPS (39). A significant difference between these studies and our own is their use of a purified exogenous TLR2 ligand and an isolated antigen to immunize mice, whereas we used an intact pathogen likely expressing more than one TLR ligand. Thus, it is possible that in vivo TLR2 signaling in response to *S. pneumoniae* is modulated by signaling inputs from other TLR, as well as non-TLR innate receptors.

Intact *S. pneumoniae* induces a mixed type 1-type 2 cytokine and antiprotein and antipolysaccharide IgG isotype response in vivo (19) that is dependent on CD4$^+$ T cells (21, 55). In both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice, we observe an intact type 2 IgG response (i.e., normal or elevated levels of IgG1) specific for both protein and polysaccharide antigens. Indeed, the normal induction of specific IgG1 in MyD88$^{-/-}$ mice in response to heat-killed *S. pneumoniae* type 14 could account for their relative resistance to subsequent killing by live *S. pneumoniae* type 14, relative to the response in naïve MyD88$^{-/-}$ mice. Additionally, the partial induction of type I IgG isotypes in MyD88$^{-/-}$ mice in response to heat-killed *S. pneumoniae* type 14 could also have contributed to protection. A protective effect of IgG1 against infection with live *S. pneumoniae* was also observed in IgG3-deficient mice immunized with an *S. pneumoniae* glycoconjugate (32). Further, mouse IgG1, IgG2b, and IgG3 monoclonal antibodies, which bound to PC epitopes on pneumococcal C-polysaccharide and shared similar or identical variable regions, provided equivalent protection against infection with *S. pneumoniae* (5). Of note, both TLR2$^{-/-}$ and MyD88$^{-/-}$ mice exhibited a normal induction of splenic IL-6 mRNA. IL-6 has been implicated in stimulating type 2 immunity (41). Further, IL-6$^{-/-}$ mice immunized with intact *S. pneumoniae* have defects in both type 1 and type 2 IgG isotypes (20). Thus, the normal IL-6 response in MyD88$^{-/-}$ mice might be critical for the intact IgG1 response to *S. pneumoniae*. The differences observed in MyD88$^{-/-}$ mice between defective in vitro induction of IL-6 protein and normal in vivo splenic IL-6-specific mRNA may reflect the engagement by *S. pneumoniae* of other innate receptors in vivo, not present in the in vitro culture, that can mediate cellular activation. Although a number of studies on pathogen-induced type 2 responses have indicated the existence of specific pathogen-derived inducers of type 2 differentiation (e.g., schistosome egg and filarial antigens) (30, 53), it remains to be determined whether *S. pneumoniae* actively induces type 2 immunity or whether this occurs through a default pathway (14).

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grants 1R01 AI49192 and 1R01 AI46551.

Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

We thank Goutam Sen, William C. Gause, and Katherine Lee (Uniformed Services University of the Health Sciences, Bethesda, Md.) for critical review of the manuscript, Andy Lees (Biosynexus, Inc., Gaithersburg, Md.) for provision of PC-KLH, and Shizuo Akira (Osaka University) and Douglas T. Golenbock (University of Massachusetts Medical School, Worcester) for providing TLR2$^{-/-}$ and MyD88$^{-/-}$ breeding pairs.

REFERENCES

1. **Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira.** 1998. Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. Immunity **9:**143–150.
2. **Akira, S., K. Takeda, and T. Kaisho.** 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. **2:**675–680.
3. **AlonsoDeVelasco, E. A., F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. **59:**591–603.
4. **Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S. S. Mambula, A. Vecchi, A. Mantovani, S. M. Levitz, and L. Romani.** 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J. Immunol. **172:**3059–3069.
5. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin.** 1984. The effects of subclass on the ability of anti-phosphocholine antibodies to protect mice from fatal infection with Streptococcus pneumoniae. J. Mol. Cell. Immunol. **1:**305–309.
6. **Colino, J., Y. Shen, and C. M. Snapper.** 2002. Dendritic cells pulsed with intact *Streptococcus pneumoniae* elicit both protein- and polysaccharide-specific immunoglobulin isotype responses in vivo through distinct mechanisms. J. Exp. Med. **195:**1–13.
7. **Colino, J., and C. M. Snapper.** 2003. Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact Streptococcus pneumoniae. J. Immunol. **171:**2354–2365.
8. **Debus, A., J. Glasner, M. Rollinghoff, and A. Gessner.** 2003. High levels of susceptibility and T helper 2 response in MyD88-deficient mice infected with *Leishmania major* are interleukin-4 dependent. Infect. Immun. **71:**7215–7218.
9. **Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. R e Sousa.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529–1531.
10. **Dillon, S., A. Agrawal, T. Van Dyke, G. Landreth, L. McCauley, A. Koh, C. Maliszewski, S. Akira, and B. Pulendran.** 2004. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-fos in dendritic cells. J. Immunol. **172:**4733–4743.
11. **Echchannaoui, H., K. Frei, C. Schnell, S. I. Leib, W. Zimmerli, and R. Landmann.** 2002. Toll-like receptor 2-deficient mice are highly suceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. J. Infect. Dis. **186:**798–806.
12. **Equils, O., M. L. Schito, H. Karahashi, Z. Madak, A. Yarali, K. S. Michelsen, A. Sher, and M. Arditi.** 2003. Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication. J. Immunol. **170:**5159–5164.
13. **Finkelman, F. D., I. M. Katona, T. Mosmann, and R. L. Coffman.** 1988. IFN-γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. J. Immunol. **140:**1022–1027.
14. **Gause, W. C., J. F. Urban, Jr., and M. J. Stadecker.** 2003. The immune response to parasitic helminths: insights from murine models. Trends Immunol. **24:**269–277.
15. **Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer.** 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. Science **303:**1526–1529.
16. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740–745.
17. **Hoebe, K., E. M. Janssen, S. O. Kim, L. Alexopoulou, R. A. Flavell, J. Han, and B. Beutler.** 2003. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat. Immunol. **4:**1223–1229.
18. **Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, K. Crozat, S. Sovath, J. Han, and B. Beutler.** 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. Nature **424:**743–748.
19. **Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira.** 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity **11:**115–122.
20. **Khan, A. Q., Y. Shen, Z. Q. Wu, T. A. Wynn, and C. M. Snapper.** 2002. Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to *Streptococcus pneumoniae*. Infect. Immun. **70:**749–761.
21. **Khan, A. Q., A. Lees, and C. M. Snapper.** 2004. Differential regulation of IgG anti-capsular polysaccharide and antiprotein responses to intact *Streptococcus pneumoniae* in the presence of cognate CD4$^+$ T cell help. J. Immunol. **172:**532–539.
22. **Knapp, S., C. W. Wieland, C. van't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll.** 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to anti-bacterial defense. J. Immunol. **172:**3132–3138.
23. **Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H.-W. Pfister, and C. J. Kirschning.** 2003. Toll-like receptor 2 participates in mediation of immune response to experimental pneumococcal meningitis. J. Immunol. **170:**438–444.
24. **Koski, G. K., K. Kariko, S. Xu, D. Weissman, P. A. Cohen, and B. J. Czerniecki.** 2004. Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that prime for high level IL-12 secretion by dendritic cells. J. Immunol. **172:**3989–3993.
25. **Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna.** 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through Toll-like receptor 9. Blood **103:**1433–1437.
26. **Latz, E., J. Franko, D. T. Golenbock, and J. R. Schreiber.** 2004. *Haemophilus influenzae* type b-outer membrane protein complex glycoconjugate vaccine induces cytokine production by engaging human Toll-like receptor 2 (TLR2) and requires the presence of TLR2 for optimal immunogenicity. J. Immunol. **172:**2431–2438.
27. **Liu, N., R. R. Montgomery, S. W. Barthold, and L. K. Bockenstedt.** 2004. Myeloid differentiation antigen 88 deficiency impairs pathogen clearance but does not alter inflammation in *Borrelia burgdorferi*-infected mice. Infect. Immun. **72:**3195–3203.
28. **Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki.** 2003. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. **198:**513–520.
29. **Luther, S. A., and J. G. Cyster.** 2001. Chemokines as regulators of T cell differentiation. Nat. Immunol. **2:**102–107.
30. **MacDonald, A. S., A. D. Straw, B. Bauman, and E. J. Pearce.** 2001. CD8$^-$ dendritic cell activation status plays an integral role in influencing Th2 response development. J. Immunol. **167:**1982–1988.
31. **Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jines, J. C. Paton, M. R. Wessels, and D. T. Golenbock.** 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc. Natl. Acad. Sci. USA **100:**1966–1971.
32. **McLay, J., E. Leonard, S. Petersen, D. Shapiro, N. S. Greenspan, and J. R. Schreiber.** 2002. γ3 gene-disrupted mice selectively deficient in the dominant subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti-polysaccharide IgG3 is corrected by induction of anti-polysaccharied IgG1. J. Immunol. **168:**3437–3443.
33. **Medzhitow, R., and C. A. Janeway, Jr.** 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell **91:**295–298.
34. **Murphy, K. M., and S. L. Reiner.** 2002. The lineage decisions of helper T cells. Nat. Rev. Immunol. **2:**933–944.

34a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academies Press, Washington, D.C.

35. **Ogunniyi, A. D., M. C. Woodrow, J. T. Poolman, and J. C. Paton.** 2001. Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. Infect. Immun. **69:**5997–6003.
36. **Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem.** 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc. Natl. Acad. Sci. USA **97:**13766–13771.
37. **Pichyangkul, S., K. Yongvanitchit, U. Kum-arb, H. Hemmi, S. Akira, A. M. Krieg, D. G. Heppner, V. A. Stewart, H. Hasegawa, S. Looareesuwan, G. D. Shanks, and R. S. Miller.** 2004. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J. Immunol. **172:**4926–4933.
38. **Pilling, P. A., M. C. Lawrence, A. M. Berry, A. D. Ogunniyi, R. A. Lock, and J. C. Paton.** 1998. Expression, purification and preliminary X-ray crystallographic analysis of PsaA, a putative metal-transporter protein of *Streptococcus pneumoniae*. Acta Crystallogr. D **54:**1464–1466.
39. **Pulendran, B., P. Kumar, C. W. Cutler, M. Mohamadzadeh, T. Van Dyke, and J. Banchereau.** 2001. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. J. Immunol. **167:**5067–5076.

40. **Redecke, V., H. Hacker, S. K. Datta, A. Fermin, P. M. Pitha, D. H. Broide, and E. Raz.** 2004. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J. Immunol. **172:**2739–2743.
41. **Rincon, M., J. Anguita, T. Nakamura, E. Fikrig, and R. A. Flavell.** 1997. Interleukin-6 directs the differentiation of IL-4-producing CD4 T cells. J. Exp. Med. **185:**461–469.
42. **Schnare, M., G. M. Barton, A. C. Holt, K. Takeda, S. Akira, and R. Medzhitov.** 2001. Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. **2:**947–950.
43. **Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning.** 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. **274:**17406–17409.
44. **Snapper, C. M., and W. E. Paul.** 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science **236:**944–947.
45. **Snapper, C. M., and F. D. Finkelman.** 1999. Immunoglobulin class switching, p. 831–861. *In* W. E. Paul (ed.), Fundamental immunology, 4th ed. Raven Press, New York, N.Y.
46. **Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta.** 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature **334:**255–258.
47. **Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira.** 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity **11:**443–451.
48. **Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa, and S. Akira.** 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int. Immunol. **12:**113–117.
49. **Takeuchi, O., K. Hoshino, and S. Akira.** 2000. TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J. Immunol. **165:**5392–5396.
50. **Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf., A. Zychhlinsky, K. Takeda, and S. Akira.** 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. **13:**933–940.
51. **Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira.** 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. **169:**10–14.
52. **Wang, Q., R. Dziarski, C. J. Kirschning, M. Muzio, and D. Gupta.** 2001. Micrococci and peptidoglycan activate TLR2–MyD88–IRAK–TRAF–NIK–IKK–NF-κB signal transduction pathway that induces transcription of interleukin-8. Infect. Immun. **69:**2270–2276.
53. **Whelan, M., M. M. Harnett, K. M. Houston, V. Patel, W. Harnett, and K. P. Rigley.** 2000. A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. J. Immunol. **164:**6453–6560.
54. **Wooten, R. M., Y. Ma, R. A. Yoder, J. P. Brown, J. H. Weis, J. F. Zachary, C. J. Kirschning, and J. J. Weis.** 2002. Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi. J. Immunol. **168:**348–355.
55. **Wu, Z.-Q., Q. Vos, Y. Shen, A. Lees, S. R. Wilson, D. E. Briles, W. C. Gause, J. J. Mond, and C. M. Snapper.** 1999. In vivo polysaccharide-specific IgG isotype responses to intact *Streptococcus pneumoniae* are T cell dependent and require CD40- and B7-ligand interactions. J. Immunol. **163:**659–667.
56. **Wu, Z. Q., A. Q. Khan, Y. Shen, J. Schartman, R. Peach, A. Lees, J. J. Mond, W. C. Gause, and C. M. Snapper.** 2000. B7 requirements for primary and secondary protein- and polysaccharide-specific Ig isotype responses to *Streptococcus pneumoniae*. J. Immunol. **165:**6840–6848.
57. **Zhang, D., G. Zhang, M. S. Hayden, M. B. Greenblatt, C. Bussey, R. A. Flavell, and S. Ghosh.** 2004. A Toll-like receptor that prevents infections by uropathogenic bacteria. Science **303:**1522–1526.

Editor: J. N. Weiser